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Time to recognize zebrafish '**affective**' behavior

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Abstract

Widely used in biomedical research, zebrafish (*Danio rerio*) are steadily gaining popularity as a model organism for studying neurobehavioral phenomena. Here, we focus on to the growing spectrum of zebrafish behavioral phenotypes and the 'bigger' biological problems these models help to address. Emphasizing the developing potential of zebrafish as a model organism in biological psychiatry, we discuss several questions related to this field: Do zebrafish have 'emotional'-like behaviors? What are their neural circuits, biomarkers, and ontogenetic origins? And, finally, how can we use this knowledge to build translational bridges to understand human emotions, motivation and personality? Representing a joint effort of several established neurobehavioral laboratories, this article outlines the mounting evidence to support emotionality in zebrafish and other fishes. This conclusion is important to justify the validity of zebrafish 'affective' paradigms and their utility for basic/translational research of normal and pathological behaviors.

Keywords

Zebrafish; biological psychiatry; fish models; behavioral syndromes; physiological correlates; emotionality

1. Introduction

Expanding the range of animal tests and model species is an important strategy in neurobehavioral research (Kalueff et al., 2007; Kalueff & Schmidt, 2011). Zebrafish (*Danio rerio*) have long been used in biomedicine, including genetics, developmental biology and toxicology. In addition, this species is currently emerging as a useful model organism for studying neurobehavioral phenomena, including normal and pathological conditions, such as cognitive, affective and substance abuse disorders (Jesuthasan, 2002; Best & Alderton, 2008; Norton et al., 2008; Stewart et al., 2010a,b). Dedicated to

various zebrafish behavioral paradigms, this special issue of '*Behaviour*' reflects the growing recognition of the potential of this organism in the field of translational biological psychiatry.

What can be learned from zebrafish in the domain of 'affective' (influenced by, or resulting from, the emotions) behaviors? To address this question, a survey was sent to various active zebrafish behavioral laboratories worldwide on behalf of the International Zebrafish Neuroscience Research Consortium (ZNRC). The main focus of this discussion was whether zebrafish have simple instinctive escape reactions, or have more complex responses representing some kind of 'emotionality'. The answer to this question is important for our ability to justify the validity of this field and establish future strategic directions of its research. This editorial article offers consolidated views of several active zebrafish laboratories, providing necessary clarification and updates in this field, and encouraging further discussion of what we know and what we have still to learn.

2. Why use zebrafish for neurobehavioral research?

This question is frequently asked by neuroscientists who use more traditional (e.g., primate or rodent) models, and want to know why to use fish instead. Institutional review boards also ask this question, aiming to understand the benefits of using the aquatic species in modeling behavioral phenomena. Various funding bodies raise these concerns when they try to decide whether a new model is worth research support. Likewise, the industry needs to decide whether zebrafish can offer useful screens for new treatments, and the general public wants to know how 'simple' fish may be useful to study complex brain phenomena, including neuropsychiatric disorders that affect the human population.

Zebrafish possess several features that make them an excellent model to study normal and pathological behaviors. First, the behaviors usually tested in laboratories (e.g., aggression, novelty exploration, anxiety-like responses) are robust, evolutionarily conserved, and shared among vertebrate species. Therefore, using fish in behavioral research is as justified as using mice or rats. The utility of zebrafish models is not limited to genetics, but includes pharmacological assays and testing various psychological stressors, similar to the use of rodent and primate models. Since zebrafish are inexpensive, small, breed in large numbers, and are easy to manipulate, this markedly reduces the costs of scientific research. Due to these practical considerations, zebrafish (along with fruit flies and worms) have significantly contributed to our understanding of how genes regulate an organism's growth, also advancing the development of powerful techniques to manipulate gene function and activity (Burne et al., 2011); however, see (Kokel & Peterson, 2008) for a balanced discussion of limitations of zebrafish paradigms.

Recently, behavioral scientists have taken a particular interest in zebrafish due to low costs, well-established powerful genetic methodologies and sophisticated (2D- and, most recently, 3D-based) video-tracking tools (Gerlai, 2005; Mathur & Guo, 2010; Cachat et al., 2011; Stewart et al., 2011). The other benefit of using zebrafish models is in enabling scientists to rapidly test a hypothesis or novel drug

treatment related to psychiatric disorders, before testing them *in-depth* in rodents or primates. Since early phenotypic characterization of novel mutations and compounds represent the main challenge in psychiatric research (Agid et al., 2007), the field can benefit from emerging zebrafish models, where behavioral responses can be quickly quantified and characterized (see, for example, how zebrafish can be used to classify drugs into functionally distinct clusters (Kokel & Peterson, 2008; Kokel et al., 2010; Rihel et al., 2010; Laggner et al., 2011)). Thus, an animal model that is low cost, genetically tractable, has a fully characterized genome, and displays robust quantifiable behaviors for which the mechanism of action can be predicted, represents a promising model for preclinical research into psychiatric disorders.

Finally, we consider attributes of the zebrafish model relevant to animal use, welfare and care (see van der Staay et al., 2009, for general discussion). The sufficient complexity, sensitivity and robustness of zebrafish behaviors make this species perfect for studying the role of experimental factors in behavioral disorders. At the same time, the simplicity of zebrafish behavioral paradigms helps reduce possible stress to the animal and time it is subjected to testing. The aquatic paradigms adhere to the 3Rs principle (de Wolf et al., 2007), as fish are less sentient than rodents, and typically use less invasive administration of drugs (i.e., immersion/inhalation vs. injections). Furthermore, unlike rodent developmental models of stress, zebrafish embryos can be exposed to neonatal stressors without having to consider the environmental effects on the mother (Teraoka et al., 1993). The availability of modern imaging and video-tracking techniques for zebrafish behavioral analyses (Egan et al., 2009; Cachat et al., 2010b; Cachat et al., 2011; Blaser & Rosemberg, 2012) enables researchers to obtain data from fewer animals, or to obtain more information per animal, thereby reducing future use of animals. Thus, the adaption of zebrafish models for basic research embodies the core objective of ethical research to advance scientific knowledge while following the 3Rs principles (Guide for the care and use of laboratory animals, 2011).

3. How to identify emotions/emotionality in animals, including fish?

Complex behaviors are intrinsically linked to emotional states. The first important aspect in this discussion is to agree on a definition of 'emotion'. In general, there is a distinction between *affects* (the subjective experiential-feeling component, linked to bodily events and external stimuli) and *emotions* ("closely linked to internal brain action states, triggered typically by environmental events") (Panksepp, 2005). Although the criteria for the existence of affects in animals are difficult to achieve (Panksepp, 2005), mounting evidence shows that zebrafish possess: i) neural circuits that can activate coherent fear/anxiety-like action tendencies (Northcutt, 1995; Maximino & Herculano, 2010; Jesuthasan, 2012; Lau et al., 2011); ii) sensitivity of such circuits to activation or lesion in generating approach, escape, and/or avoidance responses (Agetsuma et al., 2010a; Lee et al., 2010b); and iii) key neurochemical pathways and brain activation patterns that correlate with fear or anxiety (Panula et al., 2010; Lau et al., 2011).

Conceptually, emotions are brain/mental states that give positive or negative values to a stimulus or an experience. They represent learned, acquired state or process in which the individual experience becomes meaningful because it correlates with the impact on the life of the subject. The 'emotional state' of the brain also requires some degree of attention, which can be separated from emotion, and impacts on how the stimulus is represented/used to modulate the animal's response (action) and behavior (motivation, or 'intention'). However, emotion is distinct from cognitive processes (representing the channeled information processing which builds a mental representation of the stimulus with the perception-to-action control). Rather, it influences the output of cognitive processes, e.g., representing motivation and decision-making. Furthermore, emotion can be opposed to 'innate' or 'instinctive' behaviors, which are inherent action patterns. The latter includes reflexes (e.g., the escape response (Danos & Lauder, 2012)) or non-reflex actions (i.e., actions requiring brain processing, such as drug-induced place-preference (Mathur et al., 2011)). Such generalized reflexive 'hard-wired' responses to stimuli can be performed without the animal needing to recognize the stimuli generating the response (Bekoff, 2000).

The definition of *fear* and *anxiety* is also critical when asking whether zebrafish have such emotions. Anxiety can be defined as an emotional state in which the animal displays a strong fear response to what would normally be neutral or mild stimuli (Jesuthasan, 2012). Zebrafish can clearly enter such a state. For example, Jesuthasan's group studying helpless behavior (the failure to learn avoidance after exposure to a strong stressor) showed that fish possess more than 'primitive' escape responses. Like humans and other mammals, their future behavior can be rendered maladaptive by extreme stress (Lee et al., 2010a) – a conclusion to which another group also came, reporting impaired learning following acute predator exposure (Gaikwad et al., 2011). Likewise, anxiety and fear can be distinguished on the basis of timing and direction of the animal's response relative to the threat. Fear is characterized by moving *away* from a *current* threat, whereas anxiety often occurs when there is movement *towards* a *potential* threat. Thus, while escape is likely to represent a fear response, zebrafish are clearly capable of more than this, and their complex anxiety-like behavior includes freezing, erratic movements, hypolocomotion, geo-, thigmo- and scototaxis (Grossman et al., 2010; Maximino et al., 2010a; Cachat et al., 2011; Stewart et al., 2011).

Finally, as early research on emotion faltered due to the focus on subjective experience associated with emotion, i.e., conscious awareness or thought (LeDoux, 2000), rapid progress in understanding emotionality was possible only when subjective aspects ('feelings') were no longer used as the measure of the emotion. Thus, an emotion is not a subjective state, and should not be conflated with 'feelings' (the *subjective* experience of emotion). While feelings are impossible to determine in zebrafish (or mice and other non-human animals), objective criteria (e.g., startle, freezing, cortisol levels or genomic responses) have proven to be useful biomarkers of emotional states. The knowledge gained from experiments using these criteria, such as the discovery of the involvement of the amygdala and habenula, has been shown to be highly relevant to humans. For example, human patients with damage to the

amygdala have strongly reduced feeling of fear, fully consistent with early animal studies where no subjective feelings were considered (LaBar & LeDoux, 1996; Adolphs et al., 2005).

4. If emotion is not unique to humans, can fish have it?

Importantly, emotion is not unique to humans (Bekoff, 2000), although the degree to which it is experienced and can be measured, is debatable. First, it is unlikely that emotion first appeared in humans with no evolutionary precursor in animals, especially given the common brain systems and chemicals underlying emotion shared among humans and animals (Bekoff, 2000). The physiological and behavioral correlates of emotional responses in humans offer candidate indirect measures of emotional responses in animals. Although non-human mammals have been the subject of this research, extending it to emotional experiences in fish is still limited (Braithwaite & Boulcott, 2007). Nevertheless, there is no reason to presume that zebrafish do not experience something akin to emotion. For example, fish can experience fear, pain (Sneddon et al., 2003a,b) and, presumably, *pleasure* (e.g., from successful foraging and reproductive behaviors). Fish can also anticipate positive and negative stimuli through time-place learning or other forms of associative learning (Reebs, 1993; Ferrari et al., 2010) with the resulting emotional states of avoidance or preference (Darland & Dowling, 2001). Whether these states can be called 'dread' or 'hope' is still not fully recognized by the scientific community. For example, a recent paper studying 'hope' in cichlids (Wisenden et al., 2008) resulted in the authors being forced to remove the word 'hope' from the manuscript (now informally referred to as the 'hopeless' study). It also remains unclear whether individuals can infer a danger/fear or a reward from the behavioral expression of emotion of other individuals. Nevertheless, given the detailed knowledge accumulating on zebrafish cognitive and affective processes, zebrafish are becoming a very good candidate for exploring the behavioral and physiological expression of emotion-like states in fish.

Notably, positive or negative values vary between individuals, depending on their personal experience and learning. Positive and negative emotions are built through the activation of highly conserved neural circuits (mostly subcortical/subpallial) and neuromodulatory systems (e.g., monoamines, acetylcholine and neuropeptides) in both zebrafish and mammals. Convincing zebrafish evidence includes the role of the habenular/interpeduncular circuitry in fear responses, since abrogating this connectivity prevents adaptation to a conditioned stimulus by flight in a fear-conditioning paradigm (Agetsuma et al., 2010b). In line with this, the role of dopamine in attributing positive emotional values has been shown in zebrafish, since conditioned place preference can be induced by amphetamines directly activating the dopaminergic system (Ninkovic et al., 2006), or by morphine, in a manner dependent on dopamine receptors (Bretaud et al., 2007). This relates to emotion as the brain is initially trained through experience (e.g., food ingestion) to translate an activation of the reward system into positive values. The mechanisms underlying positive and negative emotional values are further based in the visceral afferents of the

autonomic nervous system, largely recognized as a physiological determinant, or at least covariate, of affect and emotion in vertebrates (Porges, 1997).

5. Are emotions predicated on being self-aware or motivated?

While it appears that fish display some form of emotional behavior, the question of how much of a conscious experience fish, is debatable. Albeit relevant for animal care, this question has little conceptual bearing on using zebrafish for studies of emotions in the context of modeling affective states. Are emotions predicated on being self aware? Not necessarily, since emotions represent physiological means to amplify a response to an innate or acquired cue. Across the animal kingdom, animals have innate pathways (determined by various genetic factors) that lead to behavioral responses. A good example for this is fear, which can be a reflex to an assumed threat (e.g., alarm cue released from damaged cells in the epithelium evoking fright response in zebrafish (Speedie & Gerlai, 2008)). However, such reactions can be coupled with other events. For example, if fright is paired with a specific visual cue previously not associated with fear, zebrafish will learn to react to the visual cue alone (Hall & Suboski, 1995), becoming anxious in response to something they learned. Thus, how the fish 'feel' is changed based on an experience, and this 'feeling' is behaviorally expressed.

Another evidence in support of zebrafish emotionality is that fish have the covariation of different behaviors, forming the basis for their personality (Dingemanse & Réale, 2005; Dingemanse et al., 2009; Wilson et al., 2011). For example, across fish taxa, individual fish exhibit differences in aggressive behaviors or boldness, which may be due to brain developmental (Reddon & Hurd, 2008), experience-related (Dingemanse et al., 2009) or maternal effects (Wisenden et al., 2012). In humans, aggressive behaviors have the potential to evoke emotional responses in others in the same locale. Similarly, fish personality traits, when expressed, have the potential to evoke changes in other individuals. Thus, while zebrafish may lack the ability to analyze their emotions *post-hoc*, or to temper them cognitively like humans, it is clear that the neurotransmitters, neurological pathways, memory and diverse behaviors typically associated with emotionality, are all present in zebrafish.

The role of motivation is another question inherently linked to the problem of emotionality in zebrafish. Consider, for example, the internal states that control the magnitude and direction of voluntary behavior, such as foraging, mating or predator avoidance. Motivated behaviors can be regulated by physiological states (e.g., feeding), anticipatory mechanisms (e.g., behaviors at dawn or dusk, mating, sleeping, operant learned behavior) and hedonic rewards (e.g., conditioned place preferences). Fish also display anxiety-like behavior (see above), avoiding anxious contexts or stimuli. Since zebrafish are highly social fish, social motivation also affect their behavior, for example, with the sight of conspecific serving as a strong positive reinforcer (Saverino & Gerlai, 2008). Finally, recent studies (Sneddon et al., 2003a,b) suggests that fish can be 'aware' of pain (Stockinger, 2011), which is also linked to specific motivational

states. Together, these characteristics help define motivational behavior for zebrafish, paralleling the kinds of human emotions that are currently recognized and accepted.

6. Zebrafish 'emotions': where, how and when?

A key question in fish behavioral research is *where* in the brain and *how* exactly (i.e., via which neurotransmitters and circuits) the individual emotional states are encoded? For example, in early active avoidance learning studies using goldfish, acquisition and retention of conditioned avoidance response (flight) was prevented by ablation of the dorsomedial pallium (Portavella et al., 2004), a structure hypothesized (based on anatomical and gene expression data) to be homologous to the mammalian amygdala (Northcutt, 1995; Mueller et al., 2011). As recent data (Portavella et al., 2004; Salas et al., 2006) add functional support to this hypothesis, other teleost fish as well as zebrafish can further extend the generality of these findings.

Importantly, the understanding of *how* emotion is generated by zebrafish brain can occur at several levels – from the neural circuitry involved (e.g., neural and glial cells, the sensory system, the motor output neurons and stress circuitry), to the characterization of how information is transmitted and stored in the network (Jesuthasan, 2012). However, while homologous structures occupy different locations in teleost and mammalian brains, the identification of similar connectivity and function between species presents a considerable challenge. For example, the teleost amygdala resides in the medial region of the dorsal pallium, following a developmental trajectory quite distinct from tetrapod brains (Wullimann & Mueller, 2004). Does this prevent fish from having affective behaviors? Clearly not – rather, greater advantage may be found in utilizing zebrafish to discover new facets about the neural circuits underlying fear and anxiety, and how they evolved in different species. The same tools proven to be useful in rodents, can be applied efficiently for this task in zebrafish, including imaging of neural activity using calcium indicators or mapping the expression of immediate early genes, such as *c-fos* (Jesuthasan, 2012).

Another highly relevant question is when zebrafish emotions emerge. As already mentioned, there is a growing evidence for emotion in adult zebrafish. Zebrafish are easily trainable by rewarding and aversive conditioning, and remain trained over an extended period of time, arguing against simple reflexive behavioral responses. However, the question as to when emotions emerge, and what is the role, if any, of epigenetic effects, has received little attention in behavioral experiments. For example, although larval (e.g., 5 day old) zebrafish show habituation, there is little evidence for them to be trainable (see, however, recent reports on conditioning in larval fish (Lee et al., 2010b)). Hence, we can expect the emergence of emotions at later larval stages. The anatomical maturation of the emotional brain (e.g., the dorsal telencephalic region) has not been thoroughly studied in zebrafish. Similarly, the emergence of behaviors during larval maturation has not been addressed in behavioral studies, representing another important direction for future studies in this field. For example, the dopaminergic (D1/D2 receptor) transmission was found in the zebrafish brain between 3-5 dpf (early larval period), including the D2

receptor modulation of simple motor behavior in larvae (Souza et al., 2011). Thus, some of the central neutral circuitry likely controlling affective behavior may already be in place at the onset of the larval stage, suggesting that affective behaviors and motivation can develop in zebrafish rather early.

7. Conceptual challenges

Avoiding anthropomorphic interpretations is a common concern when developing experimental (animal) models of brain states (Holmes, 2003; Wynne, 2004). The progress in the study of animal emotions has been significantly hindered due to fear of being 'nonscientific', and by the argument that we cannot understand animals and their own perspectives because we are not 'one of them' (Allen & Bekoff, 1999; Bekoff, 2000). This, and the risk of funding bodies, regulating authorities and the public questioning the validity of studying emotion in zebrafish, leads to the noticeable avoidance of the term 'emotionality' in animal research, despite the growing evidence for the neural components and behavioral manifestations of emotion-like states in fishes.

In general, emotions can be correlated to more or less sophisticated perceptions, actions, strategies or thoughts, depending on the cognitive capabilities and skills of the species as well as individuals within species. Therefore, the fundamental question for researchers is how emotion and cognition are linked, and how emotions are felt or reflected on? Moreover, which species possess the capacity to engage in reflection, and to what extent? (see (Bekoff, 2000) for discussion). When interpreting zebrafish behavior, one challenge is dissecting learning from affective responses, to ensure that complex 'emotional' behaviors are not merely conditioned reflexes (e.g., based on pairing a stimulus with pain or distress). A second evidence to support the idea of zebrafish emotionality is a type of motivation-driven learning in this fish species. For example, zebrafish easily become addicted to psychostimulants, such as cocaine (Darland & Dowling, 2001). Humans use stimulants to modify mood, which ultimately means that we elect to alter how we process information from internal or external sources. While addicted zebrafish may not be cognizant of the cost/benefit process they are engaged in, they clearly respond in the same manner. In line with this, zebrafish demonstrate the same anxious states during withdrawal from cocaine (López-Patiño et al., 2008) or other drugs of abuse (Cachat et al., 2010a).

8. Concluding remarks

Despite the advancements made by mammalian models toward understanding normal behavior and psychiatric disease, their circuitry and molecular abnormalities remain unclear (Meaney, 2010; Gama Sosa et al., 2012; Gerlai, 2012; Guo et al., 2012). By examining the factors regulating biological development, studies of larval and adult zebrafish continue to unravel the complexities of psychiatric disorders (Lieschke & Currie, 2007; Mathur & Guo, 2010; Del Bene & Wyart, 2011; Ahrens et al., 2012). Offering an important 'reference' point to decode the 'core' behavioral and physiological mechanisms

shared by humans and our evolutionarily ancestral fish species, zebrafish become a valuable addition to the existing rodent and primate models.

To fully realize the potential of zebrafish for studying brain phenomena, the comprehensive examination of their behavioral response to various manipulations is necessary (Agid et al., 2007; Cachat et al., 2011; Blaser & Rosemberg, 2012; Luca & Gerlai, 2012; Savio et al., 2012). Recent research has contributed to developing methods to reliably quantify zebrafish responses to a wide range of environmental modifiers. The repository of behavioral profiles can then be combined with well-established molecular, cellular and genetic tools to enable large-scale screening and drug discovery (Alderton et al., 2010; Rico et al., 2011; Schneider et al., 2011). The use of larval and adult zebrafish in high-throughput models is one of the most promising strategies to increase our understanding of complex psychiatric disorders (Lessman, 2011; Rihel & Schier, 2012). However, this goal is not possible without careful examination of behavioral responses (*face validity*) to known psychiatric states and treatments (*predictive validity*), identifying neural pathways (*construct validity*), individual risk factors and mechanisms of resilience (*population validity*), evaluating evolutionarily conserved traits (*homological validity*), testing replicability/reliability of zebrafish models (*internal validity*), and analyzing their relevance to 'bigger' brain phenomena (*external validity*); see (Willner, 1986; van der Staay et al., 2009; Belzung & Lemoine, 2011) for discussion of valid animal behavioral models.

Although zebrafish are increasingly used in biomedical research, mounting evidence suggests a wider application of this animal model species to biological psychiatry. The importance of zebrafish in affective research, and the growing recognition of this field, are reflected by the number of articles for the keywords 'zebrafish' and 'anxiety' (currently 74 PubMed publications from over 25 active laboratories worldwide, including 21 publications in 2011, and 20 publications in 2012; also see detailed reviews in (Blaser et al., 2010; Maximino et al., 2010b; Sackerman et al., 2010; Piato et al., 2011; Guo et al., 2012; Okamoto et al., 2012; Stewart et al., 2012)).

Finally, it is clear that the goal of our research is not to replace mammalian behavioral paradigms with zebrafish models. However, the evidence summarized here shows that translational neuroscience research benefits markedly from a complementary use of zebrafish models to understand evolutionarily conserved phenotypes, pathways and circuits (Guo et al., 2012; Okamoto et al., 2012). Together with novel techniques developed in various zebrafish laboratories and rich data collected on adult and larval behavioral phenotypes (Blaser et al., 2010; Maximino et al., 2010b; Sackerman et al., 2010; Piato et al., 2011; Guo et al., 2012; Okamoto et al., 2012; Stewart et al., 2012), this provides a significant contribution to multidisciplinary, cross-species neurobehavioral research. Therefore, it is time to move full-speed forward - from discussing whether or not fish have emotionality to addressing more important fundamental questions of zebrafish affective behaviors and their proximate mechanisms.

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